

RESEARCH ARTICLE

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# R- and S-Equol have equivalent cytoprotective effects in Friedreich's Ataxia

Timothy E Richardson<sup>1,2</sup> and James W Simpkins<sup>1\*</sup>

## Abstract

**Background:** Estradiol (E2) is a very potent cytoprotectant against a wide variety of cellular insults in numerous different cell models, including a Friedreich's ataxia (FRDA) model. Previously, we demonstrated that estrogen-like compounds are able to prevent cell death in an FRDA model independent of any known estrogen receptor (ER) by reducing reactive oxygen species (ROS) and the detrimental downstream effects of ROS buildup including oxidative damage to proteins and lipids and impaired mitochondrial function.

**Results:** We have previously demonstrated by western blot that our cell model lacks ER $\alpha$  and expresses only very low levels of ER $\beta$ . Using L-buthionine (S,R)-sulfoximine (BSO) to induce oxidative stress in human FRDA fibroblasts, we determine the potency and efficacy of the soy-derived ER $\beta$  agonist S-equol and its ER $\alpha$ -preferring enantiomer, R-equol *in vitro* on cell viability and ROS accumulation. Here we demonstrate that these equol biphenolic compounds, while significantly less potent and efficacious than E2, provide statistically similar attenuation of ROS and cytoprotection against a BSO-induced oxidative insult.

**Conclusions:** These preliminary data demonstrate that estrogen and soy-derived equols could have a beneficial effect in delaying the onset and decreasing the severity of symptoms in FRDA patients by an antioxidant mechanism. In addition, these data confirm that the protection seen previously with E2 was indeed unrelated to ER binding.

**Keywords:** Equol, 17 $\beta$ -estradiol, Antioxidants, Friedreich's Ataxia, Fibroblasts, Neuroprotection

## Background

First recognized in 1863 [1], Friedreich's Ataxia (FRDA) is the most common hereditary form of ataxia characterized by an autosomal recessive GAA trinucleotide repeat in the *FXN* gene, resulting in the absence of frataxin protein [2,3]. The exact function of frataxin is unclear, however it is necessary for iron metabolism within cells, Fe-S cluster assembly in proteins, and maintenance of cellular redox state. Without sufficient levels of frataxin, reactive oxygen species (ROS) begin to accumulate and cells are unable to maintain function of Fe-S cluster proteins essential for mitochondrial respiration leading to mitochondrial dysfunction, insufficient energy production and ultimately cell death, beginning in organs with

greater energy requirements and thus more dependent on aerobic ATP production, such as the heart, brain and spinal cord. Symptoms usually begin in the second decade of life and include ataxia, neural hearing and ocular abnormalities, scoliosis, diabetes and cardiomyopathy, which is the most common cause of premature death in FRDA patients [for review see Ref 4].

First detected in humans in 1982 [5], equol is a biphenolic isoflavone metabolized from the soy product daidzein by intestinal flora [6-8] in 14-59% of the human population [9]. Equol is known to act as an antioxidant [10,11], decreases circulating estrogens and androgens [12], inhibits DHT binding to its receptor [13] and decreases risks of prostate [9,11,14] and breast cancer [15]. Separation of racemic equol mixtures shows that S-equol binds with very high affinity to ER $\beta$  ( $K_d \sim 0.73$  nM), while its enantiomer, R-equol has a far lower affinity for ER $\beta$ , instead showing a preference for ER $\alpha$  ( $K_d \sim 15.4$  nM), while E2 has a  $K_d \sim 0.05-0.1$  nM [16,17]. These enantiomers allow for the discrimination

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between effects due to antioxidant effects and those due to ER $\beta$  activation.

We have previously shown that phenolic estrogens are able to prevent BSO-induced FRDA skin fibroblast death, as well as block the formation of ROS [18], prevent lipid peroxidation, protein damage, depletion of ATP and support the mitochondria and oxidative phosphorylation [19]. In the present study, we provide further evidence that E2 acts by an ER $\alpha$ - and ER $\beta$ -independent mechanism. In addition, we demonstrated a lack of ER $\alpha$  and a very low level of ER $\beta$  in FRDA fibroblasts by western blot [19]. Here, we show pharmacologically that ER $\beta$  is not contributing to this process, as R- and S-equol have statistically equivalent efficacies and potencies, represented here as EC<sub>50</sub> values. These data indicate that it is the phenolic ring present in the compound structure of equol and E2 and not intrinsic receptor binding ability that is responsible for cytoprotective effects in this FRDA cell model. Although these compounds are substantially less efficacious and potent than compounds previously used [18], this pharmacologic model lends support to the non-receptor mediated, non-genomic antioxidant mechanism of E2.

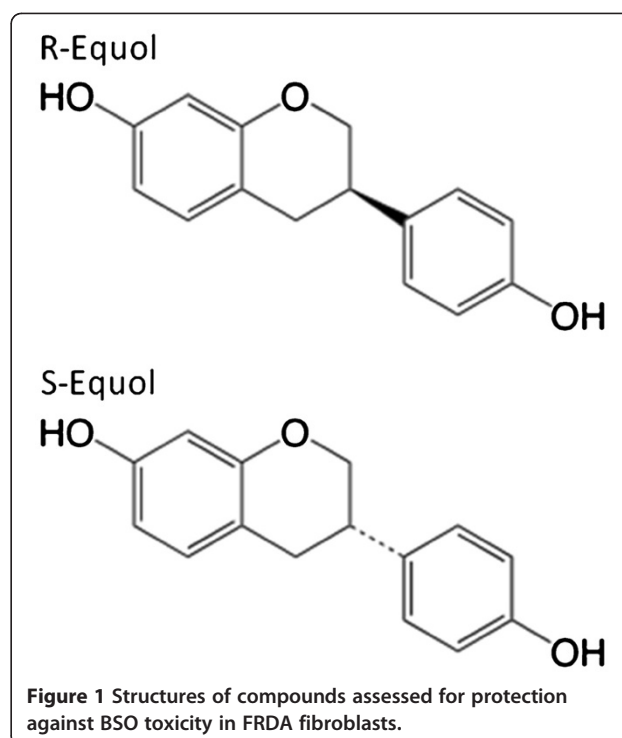
## Results

### The effects of R- and S-equol on cell viability in BSO-treated FRDA fibroblasts

To determine the effect of R- and S-equol (Figure 1) on cell viability, we first assessed their protective potential compared to 17 $\beta$ -estradiol (E2) at 100nM, a concentration previously shown to be very protective in this cell model [18]. At 100nM, both R- and S-equol provided statistically significant protection compared to the BSO-alone treated group, however the two groups did not differ significantly from each other (Figure 2a). E2 also provided significantly more protection than either of these two compounds (Figure 2a). A dose-response assessment showed that R- and S-equol have almost identical cytoprotective profiles at all concentrations (Figure 2b), and EC<sub>50</sub> evaluation demonstrated that the two have statistically equivalent EC<sub>50</sub> values (Table 1), indicating that the cytoprotective effect is not due to stimulation of ER $\beta$ .

### The effects of R- and S-equol on BSO-induced reactive oxygen species (ROS) formation

To determine the effects of R- and S-equol on ROS attenuation, these two compounds were again compared to E2 (Figure 3a). BSO induced a 2-fold increase of ROS, which was prevented by 100nM concentrations of E2, R-equol and S-equol. None of these groups differed from each other. In addition, a dose response curve for R- and S-equol shows that there is no significant difference in the ROS attenuation profiles of these two



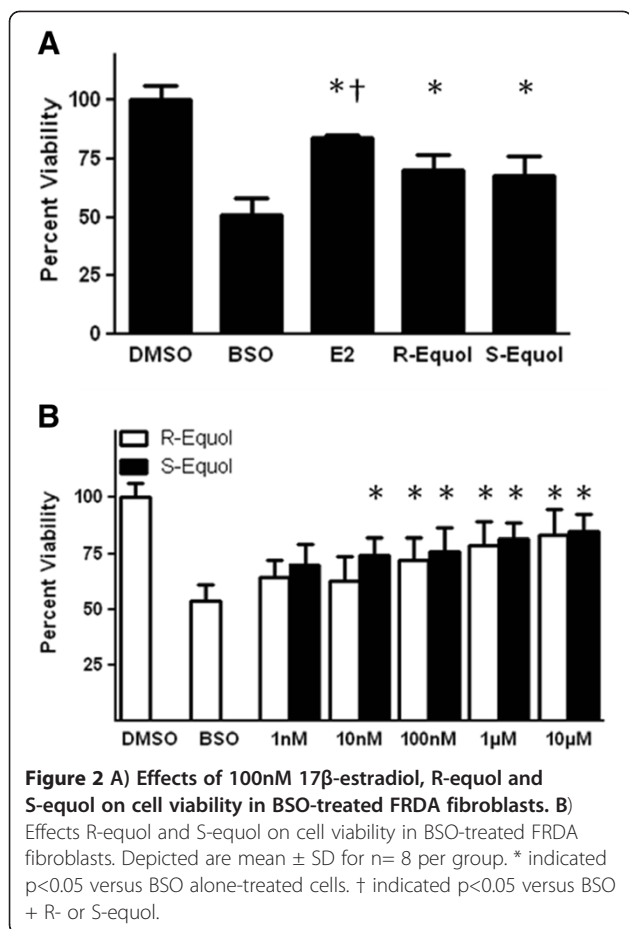
compounds at any concentration (Figure 3b), and the EC<sub>50</sub> values do not differ significantly (Table 1).

## Discussion

FRDA is the most common of the inherited ataxias world wide, affecting an estimated 1:50,000 to 1:20,000 people [2,4]. With the effective loss of functional frataxin throughout all organ systems, and the resulting ROS proliferation and mitochondrial respiration impairment, cells in organs most dependent on ATP production begin to degenerate [4,20]. This results in the loss of cells in the posterior columns and spinocerebellar tracts of the spinal cord, resulting in tremor and ataxia, as well as lateral and kyphoscoliosis, weakness, speech problems, pes cavitus, an increased incidence of diabetes mellitus and glucose intolerance and cardiac disorders, such as hypertrophic cardiomyopathy with interstitial fibrosis [4]. Disease onset

**Table 1 EC<sub>50</sub> values for R- and S-equol with respect to cell viability and ROS attenuation**

Cell Viability		
Compound	EC <sub>50</sub> (nM)	Standard Error (nM)
R-Equol	440.5	21.11
S-Equol	459.9	12.75
Reactive Oxygen species		
Compound	EC <sub>50</sub> (nM)	Standard Error (nM)
R-Equol	413.9	34.91
S-Equol	439.1	33.77



and severity is variable depending on the number of GAA trinucleotide repeats present in the first intron of the *FXN* gene, although this alone is not able to account for the full course of the disease process [21]. There is little difference between males and females in terms of disease onset, progress and severity as this is inherited in an autosomal recessive manner and symptoms begin in the first 2 decades of life, before hormone level changes in puberty [22].

Estrogen and non-feminizing estrogens have been shown to be potently cytoprotective in many different cell and animal models of disease states [23,24], including a FRDA cell model [18]. Previous observations have demonstrated that antioxidants, especially mitochondrially targeted antioxidants [25,26], including estrogen receptor agonists and non-feminizing estrogens [18] are protective against FRDA. These effects have been shown to be ER independent and are instead based in the antioxidant properties of phenolic estrogens [27,28].

Estrogens exert both genomic and non-genomic effects on redox status of cells for reviews see [29-34]. Unfortunately, no studies on the genomic effects of estrogens has been published using FRDA cells, but we have reported that these cells respond to estrogens even in the presence of a pan-estrogen receptor inhibitors, ICI 182780 [18], have no detectable ER $\alpha$

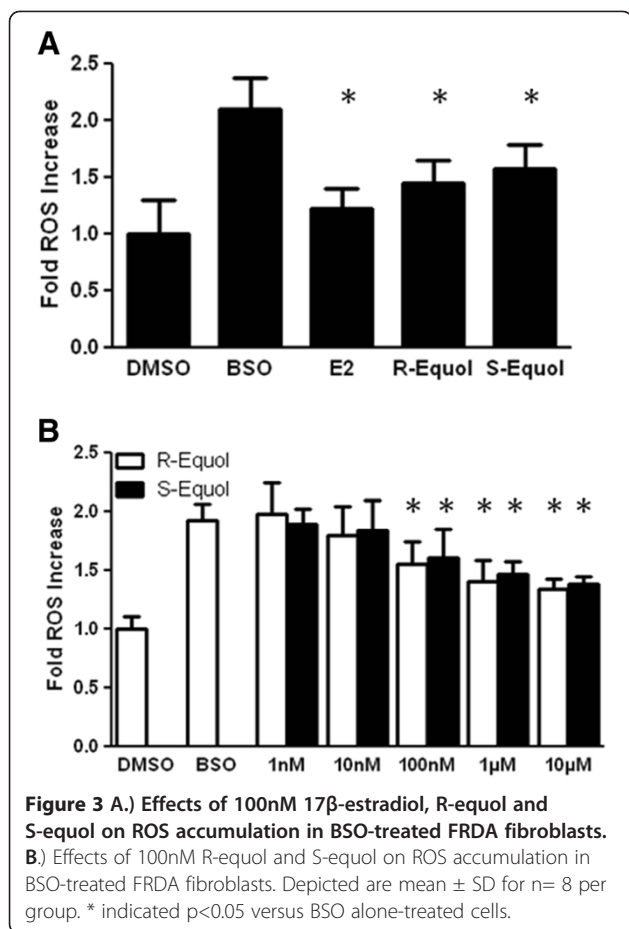
and low levels of ER $\beta$  [19], and exhibit these effects at concentrations in excess of the ED<sub>50</sub> for 17 $\beta$ -estradiol [18]. Nonetheless, genomic effects of estrogens on antioxidant enzymes have been reported, which could contribute to estrogen's antioxidant effects. For example, tamoxifen is reported to up-regulated the quinone reductase, NQO1 [29], and estrogens up-regulate expression of peroxidase-1 and MnSOD [30]. In contrast, Pajovic and Saicic [31] have reported that MnSOD, glutathione peroxidase, glutathione-S-transferase and glutathione reductase are decreased by estradiol, whereas catalase is increased. The extent to which the non-genomic effects of estrogens influence these paradoxical decreases in antioxidant enzyme expression is not known.

Estrogens are highly lipid soluble (the logarithm of the octanol/water partition coefficient, log *P*, is 3.35) and largely reside in the membrane component of cells [35] where they are ideally suited to affect oxidation of unsaturated bonds in phospholipids. Indeed, estrogens appear to intercalate into the membrane with their phenolic A ring situated near the site of lipid peroxidation [36]. We reasoned that estrogens may interrupt lipid peroxidation chain reactions via oxidation in a manner that could be redox-cycled back to the parent estrogen, using a plentiful and regenerable source of cellular reducing potential, such as glutathione or NADPH. We discovered that estrogens were converted via hydroxyl radical exposure to a quinol product that was, in turn, enzymatically reduced back to the parent estrogen in the presence of NAD(P)H as a co-factor [27,28]. This estrogen redox cycle is operative in the central nervous system [27] where it serves, together with the "classical" antioxidant mechanism for phenolic compounds, as a defense mechanism against ROS.

Equol is a naturally derived biphenolic (Figure 1) product of soy digestion in a substantial percentage of the American population [5]. It is created by intestinal flora as a racemic mixture of the R- and S-forms, with the S-form being very selective for ER $\beta$ , the only ER present in FRDA fibroblasts [19] while the R-form is only a very weak agonist at this receptor [16,17]. Our results indicate that, while not as potent or efficacious as E2 (Figure 2a and 3a) [18], the R- and S-forms of equol are equally effective in attenuating ROS (Figure 3b, Table 1) and preventing cell death (Figure 2b, Table 1). These data indicate that equol, specifically the non-feminizing R-equol, could potentially be used to prevent or delay cell death and pathologic symptoms in FRDA and supports our previous hypothesis that estrogen-like compounds are acting in a manner unrelated to any known ER [18,19].

## Conclusions

Because the biphenolic compounds R- and S-equol have statistically equal cytoprotective profiles despite



extremely different ERβ binding profiles, these data confirm that ERβ is not involved in the protective effects of E2 seen previously in this FRDA fibroblast model [18,19]. Furthermore, this study demonstrates that estrogen and soy-derived equols are effective at reducing ROS and improving cell viability in FRDA fibroblasts and shows that naturally derived soy estrogens could have a beneficial effect in delaying the onset and decreasing the severity of symptoms in FRDA patients by an antioxidant mechanism. These data add more weight to the neuroprotective hypothesis of estrogen and provide evidence that E2 and other phenol ring-containing estrogens should be considered as candidate drugs for the treatment and prevention of the symptoms of FRDA.

## Methods

### Cell culture

Fibroblasts from a 30 year old Friedreich's Ataxia (FRDA) patient (Coriell Institute, Camden NJ, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM; ThermoScientific, Waltham, MA, USA) with 10% charcoal-stripped fetal bovine serum (CSFBS; ThermoScientific, Waltham, MA, USA), 1% GlutaMAX (ThermoScientific, Waltham, MA, USA) and 1%

penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in 90% humidity and 5% CO<sub>2</sub>. At the time of treatment, the FRDA fibroblast media was changed to phenol red- and sodium pyruvate-free DMEM (ThermoScientific, Waltham, MA, USA) and 1% penicillin-streptomycin. Experiments were conducted using cell passages 15–19.

### Chemicals & reagents

17β-Estradiol (E2) was acquired from Steraloids, Inc. (Newport, RI, USA). L-buthionine (S,R)-sulfoximine (BSO) was obtained from Sigma-Aldrich (St Louis, MO, USA). R- and S- Equol were obtained from the laboratory of Dr Robert J Handa at The University of Arizona.

### Treatment paradigm

FRDA fibroblasts were removed from culture with 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) and plated on 96-well plates at a density of 3000 cells per well in DMEM with 10% CSFBS, 1% GlutaMAX and 1% penicillin-streptomycin. After 24 hours the media was removed and replaced with phenol red- and sodium pyruvate-free DMEM with 1% penicillin-streptomycin. The cells were then treated for 12 to 48 hours with either dimethyl sulfide vehicle control (DMSO; Sigma-Aldrich, St Louis, MO, USA) or 1mM BSO in the presence of E2, R-equol or S-equol ((3S)-3-(4-Hydroxyphenyl)-7-chromanol). This duration of exposure was chosen based on our observation of BSO-induced enhancement of ROS at 12 hours and cell death at 48 hours [18].

### Calcein AM cell viability assay

Cells were plated on a 96-well plate at a density of 5,000 cells per well, then treated with vehicle or 1mM BSO. After 48 hours of BSO treatment, the media was removed, and 1 μg/mL Calcein AM (CalBiochem, San Diego, CA, USA) in phosphate buffer pH 7.2 (PBS; Fisher Scientific, Pittsburg, PA, USA) was added to each well and the plate was incubated for 10 minutes at 37°C. Cell viability was determined with a Tecan Infinite M200 (Tecan Systems, Inc., San Jose, CA) plate reader with an excitation of 490nm and emission of 520nm at 48 hours.

### Reactive oxygen species assay

After 12 hours of treatment the media was removed from each well of the 96-well plate, and 100μL of a 1μM 2',7'-Dichlorodihydrofluorescein diacetate (DCFDA; AnaSpec Inc., Fremont, CA, USA) in PBS was added to each well. The plates were returned to a 37°C incubator for 20 minutes, then each well was washed three times with PBS and the resulting reaction was read on a Tecan Infinite M200 plate reader with an absorbance of 495 nm and an emission of 529 nm.



## Data and statistics

All data are displayed as mean  $\pm$  1 standard deviation. These data were analyzed using the ANOVA against an alpha level of 0.05. All bar graphs were made using GraphPad Prism 5 and EC<sub>50</sub> calculations were made with GraphPad Prism 5. For all groups, n=8 wells and experiments were repeated three times to ensure consistency.

## Abbreviations

BSO: L-buthionine (S,R)-sulfoximine; FRDA: Friedreich's Ataxia; E2: 17 $\beta$ -Estradiol; ROS: Reactive oxygen species; ER: Estrogen receptor; ER $\alpha$ : Estrogen receptor  $\alpha$ ; ER $\beta$ : Estrogen receptor  $\beta$ .

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

TER carried out the experiments, performed statistical analysis and wrote the initial draft of the manuscript. JWS revised and approved the final manuscript. All authors were involved with the conception and design of the studies. Both authors read and approved the final manuscript.

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